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FRACTIONATION BY SUCROSE DENSITY GRADIENT CENTRIFUGATION OF MEMBRANE FRAGMENTS DERIVED BY SONIC DISRUPTION OF BEEF HEART MITOCHONDRIA

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SUMMARY

1. Fractionation of beef heart submitochondrial membrane fragments derived by sonic disruption was achieved by linear sucrose density gradient centrifugation. Three distinct membrane fractions were obtained: a pellet (P), a middle (X) and an upper (Y) zone.

2. Electron micrographs revealed that the pellet contained large single pieces and aggregates of membrane fragments. The X zone contained rough membrane fragments with protruding subunits (F_1) and the Y zone contained relatively small fragments without subunits.

3. In comparison with the Y zone, the X zone exhibited higher activities of NADH oxidase and ATPase, higher oligomycin-induced respiratory control and cytochrome content, but lower activities of NADH:cytochrome *c* reductase (rotenone-sensitive) and cytochrome oxidase with externally-added cytochrome *c* as the substrate. Lubrol greatly enhanced the cytochrome oxidase activity in the X zone but not in the Y zone. NADH oxidase was greatly stimulated by externally added cytochrome *c* in the Y zone but not in the X zone. In general, the pellet exhibited varying activities falling between those of the X zone and the Y zone.

4. The relationship between these membrane fractions and the conventional EDTA particle preparation is discussed.

INTRODUCTION

The apparent asymmetry of the mitochondrial inner membrane as revealed (*cf.* ref. 1) recently from studies on reconstitution, ion translocation, and probe responses emphasizes the intimate relationship between the structure and functions of the mitochondrial inner membrane. Submitochondrial particle preparations derived by sonic disruption of intact mitochondria have been proven in recent years to be a useful tool for the study of the respiratory chain-linked functions especially those

Abbreviations: ANS, 8-anilinonaphthalene 1-sulfonate; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

involved in energy coupling. It is generally accepted that these particle preparations represent membrane fragments which are "inside out" vesicles[2] with an inverted membrane polarity in relation to intact mitochondria. However, morphological heterogeneity of submitochondrial preparations has been reported by Malviya *et al.*[3] and Stasny and Crane[4]. In order to quantitize the structural and functional relationship a more defined and homogeneous membrane preparation is, therefore, essential.

This paper describes a method of linear sucrose density gradient centrifugation to fractionate the membrane fragments derived from beef heart mitochondria by sonication. Three distinct membrane fractions have been obtained and characterized with respect to their morphological and biochemical properties. The relationship between these membrane fractions and the conventional EDTA particle preparation is also discussed. Part of this work has been presented previously[5].

MATERIALS AND METHODS

Chemicals

Antimycin A, cytochrome *c* (Type VI, from horse heart), oligomycin and rotenone were obtained from Sigma Chemical Co. 8-Anilinonaphthalene 1-sulfonate (ANS) obtained commercially was purified as described previously[6]. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) was a gift of Dr P. Heytler, E. I. duPont de Nemours and Co., Wilmington, Del. All other chemicals are of the purest grade available commercially. Glass-redistilled water was used throughout the present study.

Preparation of mitochondria and sonic fragments

Heavy beef heart mitochondria were isolated according to Löw and Vallin[7]. EDTA particles were prepared by sonication in the presence of 2 mM EDTA (pH 8.6–8.7), essentially as described by Lee and Ernster[8]. 8 ml of frozen mitochondria (40–45 mg protein/ml) were thawed and diluted with 10 ml of 0.25 M sucrose and 0.2 ml of 0.2 M EDTA, pH 7.5. The mitochondrial suspension was adjusted to pH 8.6–8.7 with 0.1 M NaOH and brought up to a final volume of 20 ml with 0.25 M sucrose. The mitochondrial suspension was then subjected to sonic oscillation for 35 s at an output of 65 W with a Branson Sonifier (Model W-185) while being cooled in an ice–water bath. The sonicated suspension was centrifuged in a Sorvall SS-34 rotor at 9000 rev./min ($9750 \times g$) for 10 min. The supernatant thus obtained was then used as the starting material for the density gradient centrifugation.

Linear density gradient centrifugation

The linear density gradient was obtained by mixing 17 ml of 0.6 M (19.2%, by weight) and 17 ml of 1.3 M (38.4%, by weight) sucrose solution in a density gradient mixing chamber. The gradient was then stored overnight at 4 °C. Immediately before the centrifugation, 5 ml of the starting material were layered on the top of the gradient. The gradient was then placed in a swinging bucket rotor (SW 27, Beckman) and was centrifuged at 22000 rev./min (average $63000 \times g$) in a Beckman L2 65B ultracentrifuge for 3 h with the brake off. After centrifugation, the gradient was collected in 1-ml fractions by inserting a syringe needle in the side of the gradient tube approximately 3.3 cm from the bottom to avoid contamination by the pellet portion. To facilitate the collection, pressure was applied to the top of the tube.

Electron microscopic studies

Negative staining was carried out with 2% phosphotungstate (pH 7.4). Five samples were examined: the starting material, the pellet (P), the X zone, the Y zone and the supernatant (S). Copper grids of 400 mesh, covered with a Formvar carbon film, were used. The samples were diluted several times with a medium consisting of 0.20 M sucrose and 30 mM Tris–acetate, pH 7.5. One drop of the diluted sample was deposited on the grids and was left in contact for 1 min. The excess was removed with filter paper and the grid was covered with a drop of phosphotungststate. After 1 min of contact, the excess solution was removed and the grid was allowed to dry. For each sample several dilutions were made and the grids were examined with either an AEI electron microscope at 60 kV with a 50-μm aperture or a JEOL (JEM8T) at 60 kV with a 50-μm aperture at a magnification of 20000× to 60000×.

Difference spectra of cytochromes and cytochrome content

Spectra of reduced *minus* oxidized pigments of the respiratory chain were determined by means of a wavelength scanning spectrophotometer[9,10] using the trapped steady-state technique of Chance and Spencer[11] as further described by Chance and Schoener[12]. Concentrations of respiratory chain pigments were calculated by the use of the following room temperature millimolar extinction coefficients and the low temperature absorption enhancement factors. The millimolar extinction coefficients were: cytochrome $a+a_3$ (605–630), 24 (ref. 13); cytochrome b (562–575), 20 (ref. 14); and cytochrome c (550–540), 19.1 (ref. 15). The absorption enhancement factors[16] were 6, 10, and 8 for cytochromes $a+a_3$, b and c, respectively.

Assays

The detailed conditions of all the assays are described in their respective figure and table legends. Protein was determined by the method of Lowry *et al.*[17].

NADH oxidase activity (NADH:O_2 oxidoreductase) was assayed spectrophotometrically by following the decrease in absorbance of NADH at 340 nm.

ANS fluorescence was measured with an Hitachi MPF-2A spectrofluorimeter using 380 nm for excitation and 480 nm for emission as described by Lee[18].

ATPase activity (ATP phosphohydrolase, EC 3.6.1.4) was assayed by the amount of inorganic phosphate released from the hydrolysis of ATP. Inorganic phosphate was determined according to the method of Lindberg and Ernster[19].

NADH:cytochrome c reductase activity (NADH:cytochrome c oxidoreductase, EC 1.6.2.1) was assayed spectrophotometrically by following the reduction of cytochrome c at 550 nm in the presence of KCN, as described by Sottocasa *et al.*[20].

Monoamine oxidase activity (monoamine:O_2 oxidoreductase, EC 1.4.3.4) was assayed with a modified spectrophotometric method of Tabor *et al.*[21] as described by Schnaitman and Greenawalt[22].

Cytochrome c oxidase activity (cytochrome c:O_2 oxidoreductase, EC 1.9.3.1) was assayed by following the oxidation of reduced cytochrome c at 550 nm, as described by Sottocasa *et al.*[20].

Malate dehydrogenase activity (L-malate:NAD^+ oxidoreductase, EC 1.1.1.37) was assayed according to Englard and Breiger[23] by following the decrease in absorbance of NADH at 340 nm in the presence of rotenone to prevent the oxidation of NADH by the respiratory chain.

RESULTS

To ensure that all the membrane fragments were scanned for the study of the morphological and the biochemical properties, the sonically disrupted membrane suspension from beef heart mitochondria in the presence of EDTA, after removal of the unbroken mitochondria, was applied directly to the linear sucrose density gradient. After centrifugation, three distinguishable membrane zones and a supernatant zone (S) were observed as shown in Fig. 1. A compact pellet (P) zone was seen near the bottom of the gradient. In the middle section of the gradient there was a broad zone (X). A narrow zone (Y) was seen immediately underneath the S zone but was clearly separated from the X zone.

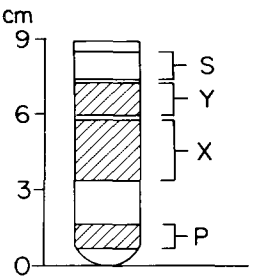

Fig. 1. Schematic representation of the density gradient illustrating the positions of a supernatant zone (S) and three membrane zones: upper zone (Y), middle zone (X) and pellet (P). The volume of the input starting material was 5 ml. The experimental details are described in Materials and Methods.

Electron microscopic studies

As shown in Fig. 2A, the starting material contained a great variety of membrane fragments of various sizes, lengths and shapes. The majority of these fragments had the protruding 80-Å subunits similar to those described as F_1 or periodic subunit structures[24–27]. The large fragments appeared to be aggregates of small ones. In the background of the Formvar one can see some small particles (80–100 Å in diameter) in either isolated or aggregated forms (Fig. 2A); most of these particles probably came from the mitochondrial matrix (*cf.* also Fig. 3D). Contamination by other membrane structures such as sarcoplasmic reticulum appeared to be negligible. The pellet (P) (Fig. 2B) appeared to contain aggregates of small fragments (500–3000 Å in length) of varying shapes and sizes. Figs 3A and B show that the X zone contained relatively homogeneous fragments with a diameter varying from 500 to 2000 Å; some membrane fragments have a size up to 6000 Å which might be formed by the aggregation of the smaller fragments during specimen preparation for electron microscope examination. More than 70% of these rough fragments had abundant spherical membrane subunits (F_1) pointing outward on the outer profiles. The diameter of these subunits was estimated to be approximately 80 Å. In this preparation only a few smooth fragments were seen (Fig. 3A). The Y zone, as shown in Fig. 3C, was composed mainly of smooth fragments (diameter between 300 and 900 Å) with no protruding subunits on the outer surface; only a few fragments (less than 5%) were found with F_1 attached. As seen in Fig. 3D, the supernatant zone (S) of the gradient

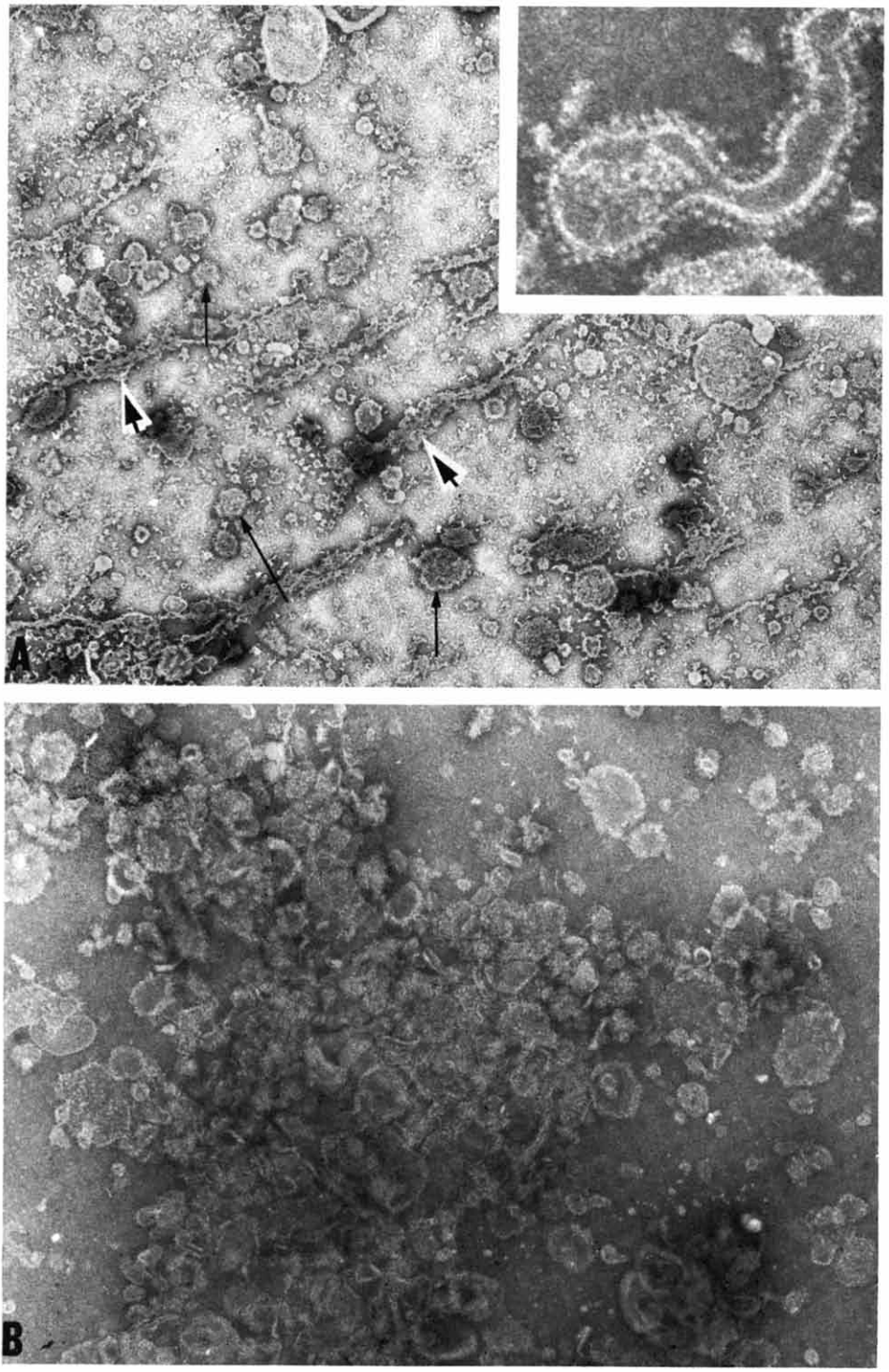

Fig. 2. Electron micrographs of negatively-stained submitochondrial membrane fragments after sonic treatment. (A) Starting material of the gradient fractionation. Thin arrows point to the vesicular fragments with subunits protruding outward. Broad arrows surrounded by a blank background point to some long ribbon-shaped membrane fragments. Magnification: ×40000. Insert (upper right corner) shows a highly magnified membrane fragment with periodic protruding subunit. Magnification: ×120000. (B) A portion of the pellet zone (P) after gradient fractionation. Magnification: ×50000.

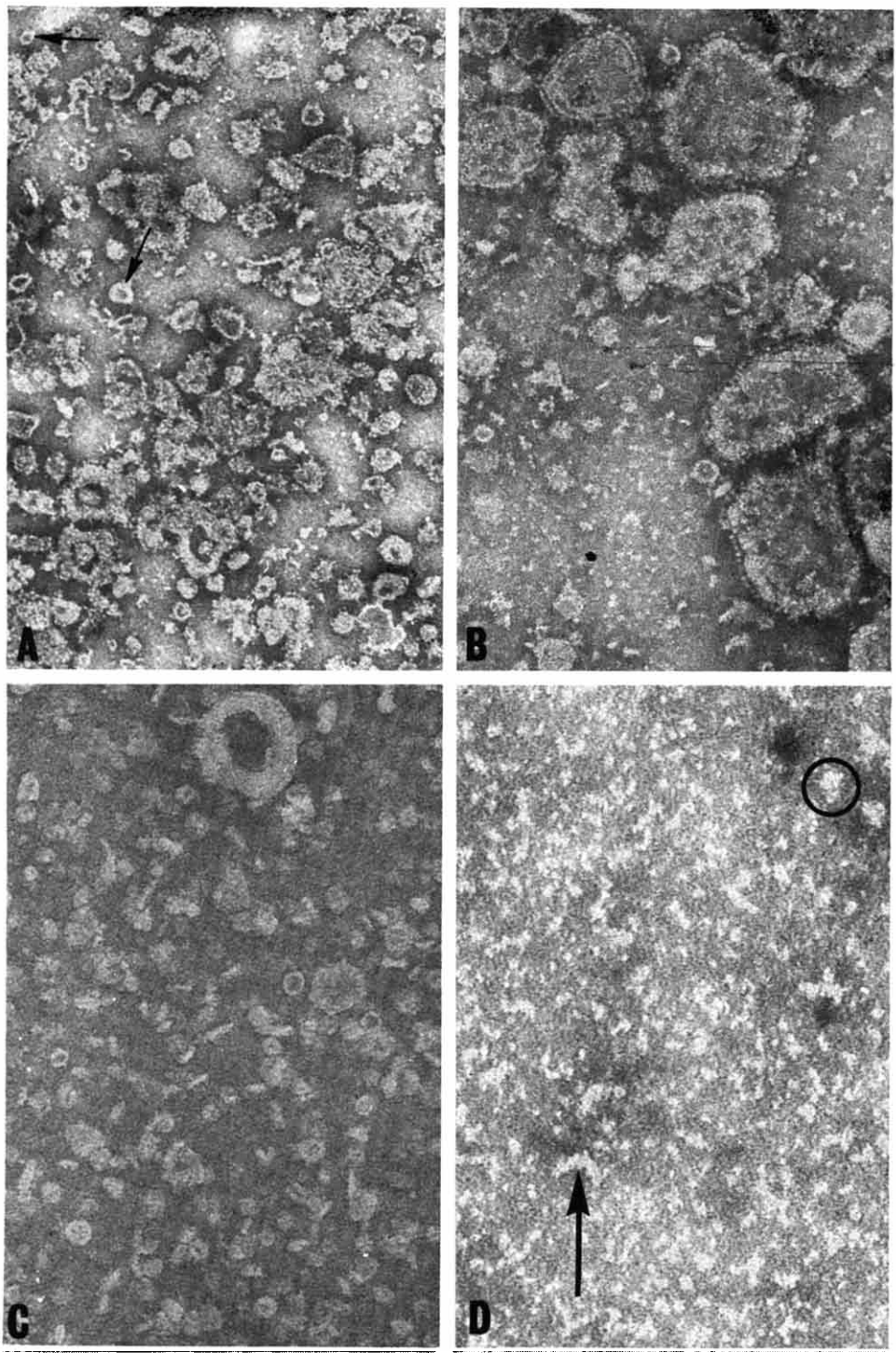

Fig. 3. Electron micrographs of negatively stained submitochondrial membrane fragments of various zones after gradient fractionation. (A) The X zone contains the rough vesicles with spherical protruding subunits. Smooth vesicles (arrows) are also seen. Magnification: × 50000. (B) The X zone at a higher magnification (× 90000). The spherical subunits are clearly seen. (C) The Y zone contains smooth vesicles. Magnification: × 80000. (D) The supernatant zone (S). Circle or arrow shows the aggregates of particles. No membrane structures are seen. Magnification: × 135000.

contained particles of 80 Å diameter in either isolated or aggregated forms. Particles of 300–500 Å diameter were also seen. Some of these particles may be components released from the mitochondrial matrix and/or detached from the membrane during sonication.

Protein profile

The protein profile of the gradient and the protein recovery of these zones are shown in Fig. 4 and Table I, respectively. The gradient was collected in 1-ml fractions except that the pellet (about 4 ml) was collected as a whole. In this experiment, the pellet (P) contained 32.5% of the total applied protein. As shown in the protein profile in Fig. 4, the pellet (P) is followed by a shoulder corresponding to the X zone which extended approximately from Fractions 15 to 27. From the Fraction 28 up, the protein content increased sharply, with a peak at Fraction 34.

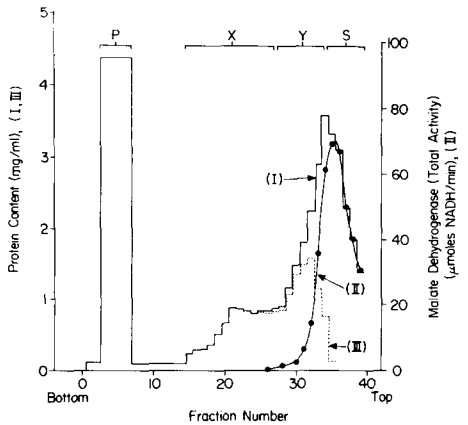

Fig. 4. Protein profiles and the total malate dehydrogenase activity. The reaction mixture for measurement of malate dehydrogenase activity consisted of 170 mM sucrose, 30 mM Tris–acetate (pH 7.5), 0.18 mM NADH, 0.83 μM rotenone and 8–32 μg protein. The reaction was started by the addition of 0.16 mM freshly prepared oxaloacetic acid. Total volume: 3 ml; temperature 25 °C. (I) Protein profile; (II) total malate dehydrogenase activity; and (III) protein profile of the Y zone after exclusion of soluble proteins as estimated from the malate dehydrogenase content.

TABLE I

PROTEIN RECOVERY IN DENSITY GRADIENT

Sample	*Protein (mg)*	*Percent*
Starting material	57.8	(100)
P	18.8	32.5
X	10.0	17.3
Y	11.8	20.4
	9.8*	16.9*
S	15.6	27.0
	17.6*	30.5*

* Based on calculation from malate dehydrogenase activity.

This large area (from Fractions 28 to 39) contained 47.4% of the total protein and covered both the Y zone (from Fractions 28 to 34) and the S zone (from Fractions 35 to 39). Several attempts were made to resolve the protein profile of the Y zone from that of the S zone, such as inserting a "filter zone" between the input sample and the gradient, or by decreasing the lowest sucrose concentration of the gradient, both of which proved fruitless.

To test the degree of contamination in the Y zone from soluble proteins which presumably originated from the S zone, the malate dehydrogenase activity was measured. The total activity profile of this enzyme is shown in Fig. 4 (Curve II). Most of the activity (up to 82.0%) was found to be confined to the S zone. Thus the contribution of soluble proteins, which presumably followed the sedimentation pattern of the malate dehydrogenase, could not account for all the protein in the Y zone. As a tentative estimation, the relatively constant specific activity of the malate dehydrogenase in Fractions 36 to 39 was used to correct for contamination by soluble proteins in the Y zone. Based on this correction, the protein profile for the Y zone free from soluble proteins was constructed (Curve III, Fig. 4). The protein thus calculated for the Y zone was 16.9% of the total, which was about 3.5% lower than that obtained by pooling Fractions 28 to 34 together (20.4%) (*cf.* Table I).

Respiratory activities

Fig. 5 shows the profile of the NADH oxidase activity measured in various metabolic states. The specific activity of NADH oxidase was highest in the X zone in the absence of oligomycin (Fig. 5, Curve I) or in the presence of oligomycin and FCCP (Fig. 5, Curve III). Essentially no activity was detected in the S zone. The effect of oligomycin, which has been demonstrated to induce respiratory control in the conventional EDTA particle preparation[2,27], is also shown (Fig. 5, Curve II). It can be seen that oligomycin greatly suppressed the respiration rate (to 1/3) in the X zone but had much less effect in the Y zone. It should be pointed out that in

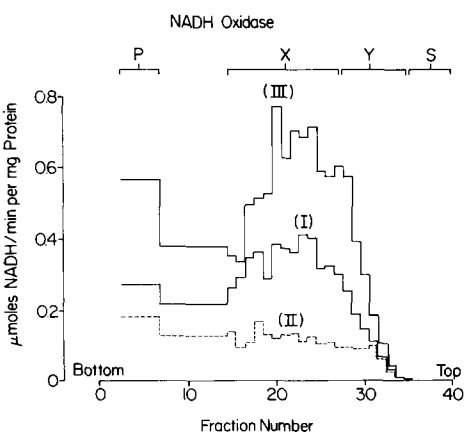

Fig. 5. NADH oxidase activity at various metabolic states. The reaction mixture consisted of 170 mM sucrose, 30 mM Tris–acetate (pH 7.5), 0.18 mM NADH. The reaction was started by the addition of enzyme (80–320 μg protein). Final volume: 3 ml; temperature 25 °C. Further addition: 2 μg oligomycin and 2 μg oligomycin + 1 μM FCCP were present in II and III, respectively.

the presence of oligomycin, the X, Y, and P zones exhibited similar residual respiratory activity (*cf.* Fig. 5, Curve II) and that none of the fractions in these zones was inhibited completely. The addition of an uncoupler, FCCP, effectively relieved the oligomycin inhibition in the X zone; a significant though small effect was also observed for the Y zone. As a consequence, the X zone exhibited the highest oligomycin-induced respiratory control. A respiratory control index of up to 7.5 was estimated (Fig. 5, Curves III and II) for Fraction 20 in this experiment.

Enhancement of ANS fluorescence

It has been shown[6,28–33] that ANS serves as a convenient reporter for the energized state of the submitochondrial membrane. As shown in Fig. 6, the X zone exhibited a high fluorescence enhancement upon addition of succinate (Fig. 6, solid line). This enhancement was greatly increased by oligomycin (Fig. 6, dotted line). A relatively poor response was seen in the Y and P zones. The fluorescence enhancement induced by succinate either alone or in combination with oligomycin was abolished by FCCP in all cases. These observations were similar to those observed on NADH oxidase (*cf.* Fig. 5).

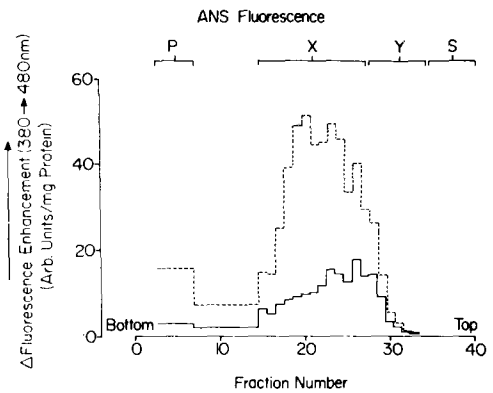

Fig. 6. ANS fluorescence response at various metabolic states. The reaction mixture consisted of 170 mM sucrose, 30 mM Tris–acetate (pH 7.5), 1.66 μM ANS, 3.3 mM succinate and 0.08–0.32 mg protein. Total volume: 3 ml; temperature 25 °C. ———, no oligomycin; – – – –, 3 μg oligomycin was present.

ATPase activities

Fig. 7 shows the ATPase activity profile of all the zones. Again, the X zone exhibited a high specific activity of ATPase (Fig. 7, solid line). The distribution of the ATPase activity closely followed that of the NADH oxidase activity (*cf.* Fig. 5). Addition of 2,4-dinitrophenol either alone or in combination with atractyloside exhibited no significant effect on the ATPase activity in any fraction. Oligomycin abolished virtually completely the ATPase activity in all cases (Fig. 7, dotted line).

NADH:cytochrome c reductase activity

All the foregoing observations seemed to indicate that the membrane fragments in the X zone were the most active in all tested activities associated with respiratory chain-linked oxidative phosphorylation, whereas those in the Y zone were the least

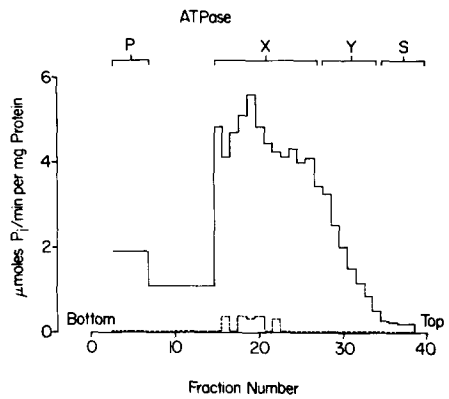

Fig. 7. ATPase activity. The reaction mixture consisted of 170 mM sucrose, 50 mM Tris–HCl (pH 7.5), 4 mM $MgSO_4$, and 0.04–0.08 mg protein in a final volume of 1 ml. The reaction was started by the addition of 5 mM ATP and terminated by the addition of 1 ml of 20% $HClO_4$ after an incubation period of 5 min at 30 °C. ———, no further addition; – – – –, 5 μg oligomycin was also present.

active. The question then arose as to the origin of the Y zone which accounted for 20.4% of the total protein, yet was relatively inactive in all reactions tested. One possibility is that the fragments in this zone originated mainly from the outer membrane of the mitochondria. Alternatively, this zone might consist of inner membrane fragments which somehow do not function properly due to some modification or damage during sonication. In order to examine these alternatives, both the rotenone-sensitive and rotenone-insensitive NADH:cytochrome *c* reductase activities were tested (Figs 8A and 8B). The Y zone had in general a higher specific activity of

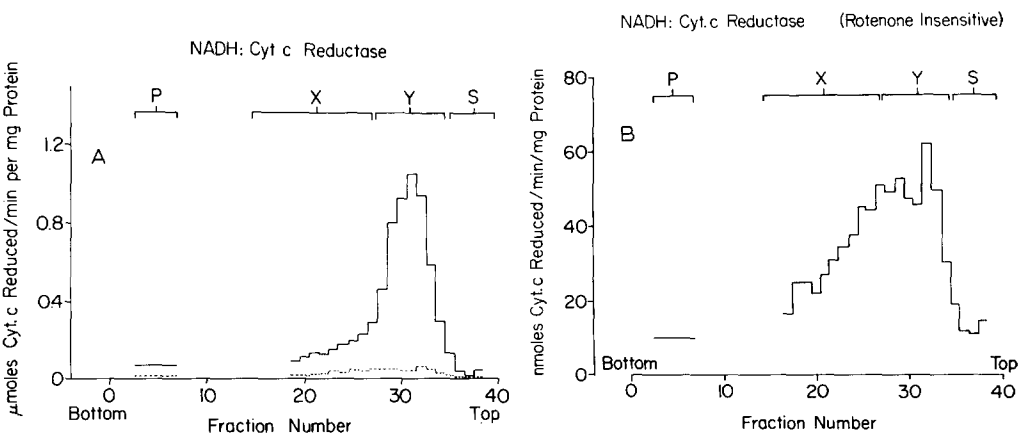

Fig. 8. NADH:cytochrome *c* reductase activity. The reaction mixture consisted of 50 mM phosphate buffer (pH 7.5), 83.3 μM cytochrome *c*, 3.3 mM KCN and 0.09–0.16 mg protein. The reaction was started upon the addition of 180 μM NADH. Total volume: 3 ml; temperature: 25 °C. When indicated, 0.83 μM rotenone was used. ——— and – – – – represent the rotenone-sensitive and -insensitive activities, respectively. B is an expanded scale of the rotenone-insensitive activity.

rotenone-insensitive NADH:cytochrome *c* reductase than other zones. No sharp peak could be detected. Furthermore, the rotenone-sensitive reductase, which was sensitive to antimycin A as well, was unexpectedly concentrated in the Y zone; the specific activity of the zone peak (Fraction 31) was a factor of 8 higher than that in the X zone. These data imply that most of the membrane fragments in the Y zone are probably of inner membrane origin with an operative NADH:cytochrome *c* reductase readily accessible to externally added cytochrome *c*.

Monoamine oxidase activity

The observation that the rotenone-insensitive NADH:cytochrome *c* reductase activity was spread over all gradient zones prompted us to study the activity of the monoamine oxidase, another marker enzyme for the outer membrane[20,22,34,35]. Table II summarizes the distribution of the activity in the gradient zones. For comparison the rotenone-insensitive NADH:cytochrome *c* reductase activity of the same preparation under similar conditions is also presented. To minimize the cross-contamination between the X and the Y zones, a new zone (M) was taken from Fractions 26 to 28. As can be seen from Table II the specific activity of both enzyme systems was the highest in the Y zone and was followed in descending order by the M, X, P and S zones. The Y zone exhibited a higher percentage (40–50%) of the total enzyme activity than of the protein (20–25%) while the reverse was true for the P zone. In the X zone the percentage of both is almost equal (10–20%). Data on the conventionally prepared EDTA submitochondrial particles from the same starting material are also presented in Table II and can be compared to those calculated for a mixture of the X and P zones (X+P).

Cytochrome c oxidase

The profile of the cytochrome *c* oxidase activity is shown in Fig. 9. The Y zone exhibited a relatively high oxidase activity, being 3 times (at zone peak) that found in the X zone (Fig. 9, Curve I). The distribution of this activity was found to be parallel to that of the rotenone-sensitive NADH:cytochrome *c* reductase (*cf.* Fig. 8). Lubrol, a nonionic detergent, had little effect on the activity in the Y zone but markedly stimulated the activity in the X zone. For instance, in the presence of 7 mg of Lubrol per mg protein, the stimulated activity in the X zone was approximately equal to that in the Y zone (Fig. 9, Curves II and III). The oxidase activity, both in the presence and absence of Lubrol, was completely blocked by KCN.

Effect of externally added cytochrome c on NADH oxidase

The effect of externally added cytochrome *c* on the NADH oxidase activity is shown in Fig. 10A. Clearly, the addition of cytochrome *c* had little effect on the X zone but significantly stimulated the NADH oxidase activity in the Y zone up to 4–6-fold at the zone peak. Cytochrome *c* at a concentration of 1.3–1.5 μM gives maximal stimulation. The magnitude of the stimulated activity in the Y zone was comparable to the activity in the X zone (*cf.* Fig. 5). It should be pointed out that in Fig. 10A only the increment in NADH oxidase activity induced by cytochrome *c* is plotted. The cytochrome *c*-stimulated NADH oxidase activity exhibited by all fractions was inhibited virtually completely by rotenone, antimycin A or KCN.

TABLE II

DISTRIBUTION OF MONOAMINE OXIDASE AND ROTENONE-INSENSITIVE NADH:CYTOCHROME *c* REDUCTASE ACTIVITIES

Monoamine oxidase: The enzyme (0.5–2.0 mg protein) was preincubated in a reaction mixture consisting of 0.15 M sucrose, 50 mM phosphate, 0.75 mg Lubrol at pH 7.5 and 38 °C for about 15 min or until a steady baseline, monitored at 250 nm, was attained. The reaction was started by the addition of 0.33 mM benzylamine. Final volume: 3 ml. Rotenone-insensitive NADH:cytochrome *c* reductase: The enzyme (0.05–0.2 mg protein) was preincubated in a reaction mixture consisting of 0.15 M sucrose, 50 mM phosphate, 120 μM cytochrome *c* and 1 μM rotenone at pH 7.5 and 38 °C for 5 min. 1.6 mM KCN was added immediately before the addition of 200 μM NADH to initiate the reaction. Final volume: 2.5 ml. Specific activity expressed as nmoles substrate/min per mg protein.

Fraction	*Expt 1*					*Expt 2*				
	Protein (%)	*Monoamine oxidase*		*NADH:cytochrome c reductase (rotenone-insensitive)*		*Protein* (%)	*Monoamine oxidase*		*NADH:cytochrome c reductase (rotenone-insensitive)*	
		Spec. act.	*% total act.*	*Spec. act.*	*% total act.*		*Spec. act.*	*% total act.*	*Spec. act.*	*% total act.*
Starting material*	(100.0)	3.03	(100.0)	47.3	(100.0)	(100.0)	3.15	(100.0)	46.7	(100.0)
S	14.3	1.12	5.3	15.9	4.8	17.6	1.42	8.0	14.2	5.3
Y	26.5	4.91	42.8	88.1	49.3	23.0	5.96	43.6	85.7	42.3
M	4.6	3.20	4.8	85.5	8.3	5.6	3.80	6.7	77.6	9.3
X	11.2	2.52	9.4	77.6	18.5	12.7	2.94	11.8	50.7	13.7
P	29.1	1.97	18.9	23.8	14.7	26.1	2.95	24.5	20.1	11.5
Sum	85.7		81.2		95.6	85.0		94.6		82.1
X+P**	40.3	2.12	28.3	38.8	33.2	38.8	2.95	36.3	30.0	25.2
ESP***	36.8	2.88	31.7	36.4	25.8	37.4	3.31	39.3	33.3	26.8

* The protein recovery for the starting material from beef heart mitochondria was 71.0% in Expt 1 and 61.6% in Expt 2. The specific activities for mitochondria were: monoamine oxidase, 3.47 and NADH:cytochrome *c* reductase, 60.7 in Expt 1; 3.92 and 68.3 in Expt 2.

** Data calculated on the basis of protein distribution.

*** EDTA submitochondrial particles prepared from the same starting material.

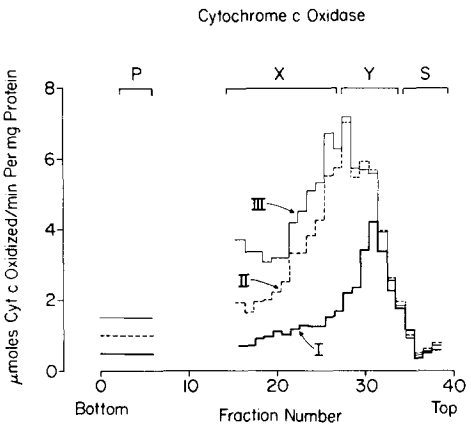

Fig. 9. Cytochrome *c* oxidase activity. The reaction mixture consisted of 50 mM phosphate buffer (pH 7.5) and 43 μM reduced cytochrome *c*. The reaction was started by the addition of 1.6–5.0 μg protein. Final volume: 3 ml; temperature: 25 °C. (I) No Lubrol; 15 μg and 30 μg Lubrol were present in II and III, respectively.

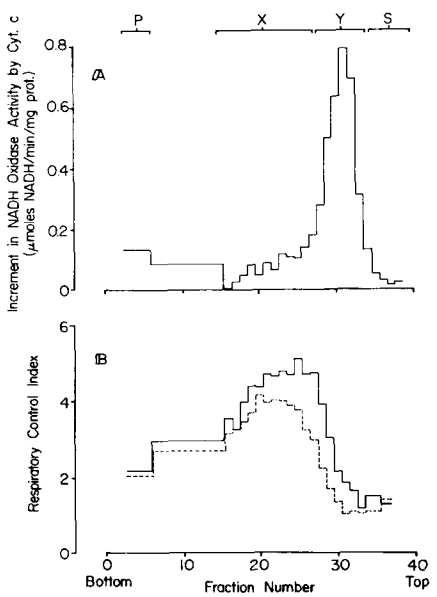

Fig. 10. Effect of externally added cytochrome *c* on the NADH oxidase activity at various metabolic states. Conditions were as described in Fig. 5. When indicated 3.3 μM cytochrome *c* was used. (A) Stimulation of NADH oxidase activity by cytochrome *c* in the absence of oligomycin and FCCP. (B) Effect of cytochrome *c* on the oligomycin-induced respiratory control. ——— and - - - - - are the respective respiratory control indexes of NADH oxidase in the absence and presence of externally added cytochrome *c*.

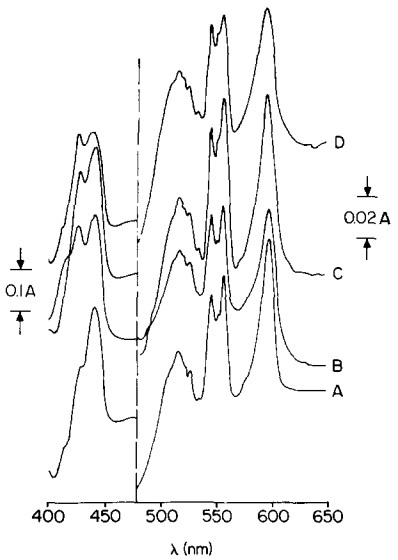

Fig. 11. The low temperature (77 °K) difference spectra (reduced − oxidized) of: A, starting material; B, pellet; C, the X zone; and D, the Y zone. The details of the measurement are given in Materials and Methods.

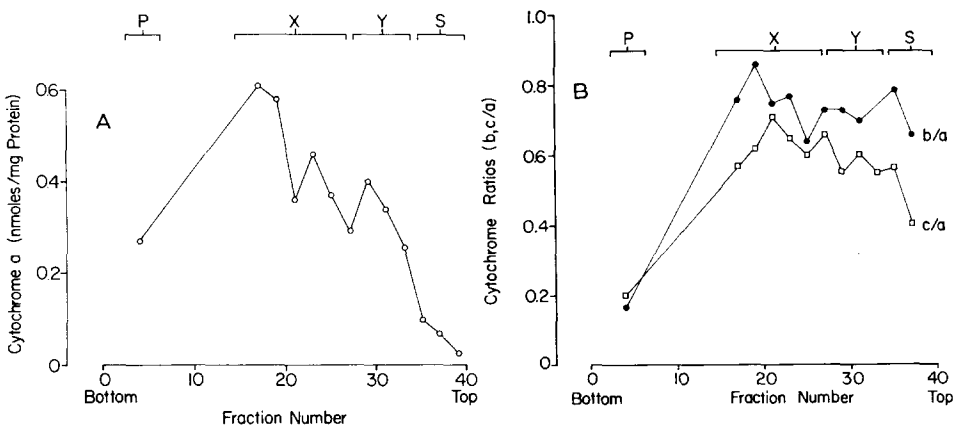

Fig. 12. The cytochrome content of the gradient zones. The details of the measurement are given in Materials and Methods. (A) Cytochrome *a* content. (B) Ratios of cytochromes *b* and *c* to cytochrome *a*.

The effect of externally added cytochrome *c* on the respiratory control index is shown in Fig. 10B. In contrast to the respiration in the X zone, the cytochrome *c*-stimulated respiration in the Y zone showed no oligomycin-induced respiratory control. The effect of cytochrome *c* was independent of the state of respiration; similar stimulated rates were obtained when cytochrome *c* was added in either the presence or absence of oligomycin ± FCCP. The increased rate of electron flow in the Y zone caused by externally added cytochrome *c* did not alter the steady-state

level of the succinoxidase-induced enhancement of ANS fluorescence in the presence or absence of oligomycin (not shown).

Cytochrome contents

The low temperature difference spectra (reduced *minus* oxidized) of the pigments of a typical fraction from each zone are shown in Fig. 11. In all fractions the cytochromes are characterized by their typical absorption peaks. The profile of cytochrome *a* content and that of the ratios of cytochromes *b*/*a* and *c*/*a* are shown in Figs 12A and 12B, respectively.

DISCUSSION

One advantage of fractionation by linear gradient over differential centrifugation is that the former approach ensures the detection of all populations of membrane fragments. In this study the sonicated membrane suspension, after removal of unbroken mitochondria, was applied directly to the gradient in order to scan all types of fragments, since it is possible that the sedimentation behavior of fragments might be altered after sedimentation by high-speed centrifugation.

The sonication of mitochondria in the presence of EDTA apparently disrupts the mitochondrial membrane and gives rise to several types of fragments seen in the X, Y and P zones in a sucrose density gradient. The F_1-encrusted vesicles in the X zone had a high cytochrome content (per mg protein) and showed high NADH oxidase and ATPase activity and a high level of energy coupling. The utilization of externally added cytochrome *c* by both NADH:cytochrome *c* reductase and cytochrome *c* oxidase was sluggish but the latter activity could be significantly stimulated by Lubrol. These observations indicate that the properties of these fragments are markedly different from those of swollen mitochondria[36] and suggest that these vesicles may represent the type of "inside out" fragments with a membrane orientation opposite to that of intact mitochondria, as first proposed by Lee and Ernster[2].

The smooth fragments in the Y zone are associated with a relatively low activity of NADH oxidase, ATPase and energy coupling. However, they exhibited a relatively high activity of both NADH:cytochrome *c* reductase and cytochrome *c* oxidase when the externally added cytochrome *c* was the substrate. Furthermore, Lubrol had little stimulatory effect on the cytochrome *c* oxidase. The addition of cytochrome *c* greatly accelerated the rate of NADH oxidation. The remarkable accessibility of these fragments to external cytochrome *c* and the ineffectiveness of Lubrol in promoting its activity are similar to the properties of swollen mitochondria[36], suggesting that these fragments may retain a membrane polarity identical to that of intact mitochondria. The lack of visible spherical subunits on the membrane in the electron micrograph and the low atractyloside-insensitive ATPase activity cannot rule out the possibility that the subunits could be enclosed inside the fragments and that these subunits are incapable of using the added ATP because of the lack of an operative adenine nucleotide translocase system[37]. The present study does not exclude the possibility that the fragments in the Y zone possess a special membrane structure with great accessibility to external cytochrome *c*, but have a membrane orientation similar to that of the X zone or no particular orientation at all.

It is worth mentioning that the activities of two marker enzyme systems for

TABLE III

ENZYMIC ACTIVITIES OF GRADIENT MEMBRANE ZONES AND OF EDTA SUBMITOCHONDRIAL PARTICLES

The activities in gradient zones are the averaged values obtained from data in Figs 5, 6, 7, 8, and 9. The data on EDTA submitochondrial particle (ESP) are taken from a typical EDTA submitochondrial particle preparation.

Fraction	*NADH oxidase (nmoles NADH/min per mg)*			*NADH:cytochrome c reductase (nmoles cytochrome c/min per mg)*		*Cytochrome c oxidase (μmoles cytochrome c/min per mg) [Lubrol (μg/assay)]*			*Fluorescence change (ANS) (arbitrary units/mg)*		*ATPase (μmoles P_i/min per mg)*
	No addition	*+ Oligomycin*	*+ Oligomycin and FCCP*	*Rotenone-sensitive*	*Rotenone-insensitive*	*0*	*15*	*30*	*– Oligomycin*	*+ Oligomycin*	
Starting material	161	114	490	237	27	1.6	2.5	2.5	2.9	14.5	1.0
Y	112	67	217	682	46	2.6	3.8	3.7	3.2	5.6	1.2
X	357	121	615	152	30	1.2	3.3	4.4	11.8	41.1	4.4
P	203	134	423	66	10	0.5	0.6	0.7	2.9	16.0	1.7
X+P	257	130	490	100	17	0.6	1.5	1.9	6.0	24.7	2.6
ESP	598	147	651	102	17	1.1	1.5	1.8	3.2	19.9	1.9

the outer membrane, the rotenone-insensitive NADH:cytochrome *c* reductase and the monoamine oxidase, are found spread over all the gradient zones. The Y zone obviously is the richest in the total activity (40–50%) of both enzymes but no sharp peaks of specific activity of the reductase (*cf.* Fig. 8) were found among fractions in the Y zone. The P and X zones contribute a significant amount of the total activity (25–35%) of both enzymes. If one employs the total activity of either enzyme as a measure of the amount of outer membrane, one comes to the conclusion that a significant amount of the outer membrane is still retained in the fractions which consist mainly of inner membrane. However, washing the frozen and thawed mitochondria prior to sonication did not alter the specific activities and the relative distribution of the monoamine oxidase in the gradient zones. The alternative possibility that some enzyme activity of both systems is of inner mitochondrial membrane origin[38] cannot be excluded.

The addition of a small amount of cytochrome *c* greatly enhanced the oxidation of NADH in the Y zone. The mode of the action of the added cytochrome *c* is not clear. The added cytochrome *c* may act through a by-pass mechanism as proposed by Camerino and Smith[39], *i.e.* the soluble cytochrome *c* is being reduced by the NADH:cytochrome *c* reductase and reoxidized by the cytochrome *c* oxidase without being incorporated into the particle preparation. This is possible since in the Y zone both enzyme systems are very active towards added cytochrome *c*. Alternatively, the added cytochrome *c* may be incorporated into the particle preparation as suggested by Tsou[40] and many others[41–44]. Nicholls *et al.*[45] have advanced the proposal that nonphosphorylating particles have two binding sites for cytochrome *c* with different affinities: a high-affinity site ($K_m = 2$–$4\ \mu$M) and a low-affinity site ($K_m = 10$–$20\ \mu$M). Since, in the Y zone, a concentration of cytochrome *c* of only 1.3–1.5 μM is required for maximal stimulation of NADH oxidation, it appears that the added cytochrome *c* may bind to the high-affinity site which Nicholls *et al.*[45] proposed to be located on the opposite site of the membrane from F_1.

Two lines of evidence led us to conclude that the conventional EDTA submitochondrial particle preparation represents a mixture of the X and the P zones of the gradient: (a) the protein recovery of the EDTA submitochondrial particle preparation covers a fraction equivalent to the sum of the P and X zones and (b) the activities of the various enzyme systems of the EDTA submitochondrial particle preparation are better accounted for by a mixture of the X and P zones (Tables II and III). Morphological heterogeneity in sonicated membrane suspensions and particle preparations has been reported[3,4]. Stasny and Crane[4] obtained two different fractions from beef heart mitochondria sonicated in water by differential centrifugation, one being deficient in and the other completely lacking subunits. Malviya *et al.*[3] examined the electron micrographs of Mg–ATP particles and estimated roughly that the vesicles with subunits were 64% and those without were 36% of the total vesicles present.

The present study indicates that fractionation by a sucrose density gradient centrifugation provides a feasible way to obtain a functionally active and a morphologically homogeneous membrane preparation such as the X zone. The availability of such a preparation would help one to study more precisely some unresolved problems which might originate from the heterogeneity of the preparation. For instance, Lee *et al.*[46] observed biphasic reduction kinetics of cytochromes *a*, *b* and *c*

when NADH was added to an oligomycin-supplemented particle suspension which was partially inhibited by KCN. Chance *et al.*[47,48] reported that ferricyanide oxidized virtually all succinate-reduced cytochromes in intact mitochondria but only 40% in EDTA particles. The elimination of this possibility is necessary for a precise interpretation of these interesting observations. Studies along these lines are currently being undertaken in our laboratory.

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